Expression of Interleukin-17 in Human Colorectal Cancer

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Abstract

Background: It is commonly acknowledged that persistent inflammation actively contributes to cancer. The role of inflammatory immunocytes and associated cytokines in the development and spread of colorectal cancer (CRC) is thought to be "double-edged." A pleiotropic proinflammatory cytokine called interleukin-17 (IL-17) can encourage inflammation brought on by cancer and shield cancer cells from immune surveillance. IL-17 is typically regarded as a promoter of CRC advancement despite disagreement. The objective of this study: To investigate the role of IL17 A as an associated factor with CRC by estimating it in tissue biopsy of colorectal cancer (CRC) patients and control using immunohistochemistry assay Patients and methods: This study was conducted from January 2022 to June 2022. Tissue biopsies were collected from a total of 35 colorectal patients, 35 of tumor-adjacent tissue and 35 biopsies of polyp tissue, from patients who had attended the Gastroenterology and Liver Diseases Hospital and 30 normal control tissue were collected from autopsies at the Forensics Medicine Department. H&E stained sections were prepared for histopathological investigation, and positively charged slides were prepared for IL-17A detection using immunohistochemistry assay. Results: It's been found that the expression of IL-17A was in the highest frequency in resection tissue (score 1), 17 cases (48.6%) and (score 2) 13 cases of malignant tissue (37.1%), followed by 12 cases (34.3%) of benign tumor tissue in (score 1) with a significant p-value (p=<0.001) in a different type of tissue. According to the staging system it was mostly expressed in patients with extensive tumor invasion (T4) and that appeared in 7 patients (43.80%) compared to patients with T1+T2+T3, with a non-significant P value (P =0.533). Conclusions: It has been found that the expression of IL17A is highly significant in CRC patients and it might have a double-edged role as its important in controlling the intestinal microbiota and getting rid of extracellular bacteria, and its pro-inflammatory effect in tumor development. IL17A expression has no significant value according to the different stages of CRC based on the TNM staging system.

1. Introduction

One of the most extensively researched carcinogenesis processes worldwide is the tumor formation in colorectal cancer (CRC) (1). Genetic changes, environmental carcinogens, and the host immune system interact intricately in CRC, ultimately leading to the unchecked development of altered cells (2). We don't fully understand the mechanisms underlying the connection between inflammation and cancer(3). T cells and other immune cells that have been activated create the proinflammatory cytokine interleukin 17 (IL17). It is a recently discovered cytokine that is crucial to both the innate and adaptive immune systems. Th-17 cells are a subset of CD4+ T helper cells that secrete IL-17 (4). In various chronic illnesses, such as inflammatory and autoimmune disorders, Th-17 causes the release of proinflammatory and neutrophil-mobilizing cytokines, mostly through IL-17 production. This study aims to estimate the (IL17A) in tissue biopsy of CRC patients and control using an immunohistochemistry assay (5).

2. Methods

Study subject: Samples collection included tissue biopsies of the tumor from patients with colorectal cancer who are a week apart from the last antibiotic dose and never had chemotherapy or radiotherapy before and the adjacent tissue, also benign tissue biopsies from endoscopic department and normal control tissue. Tumor and adjacent tissue: A total of 35 colorectal cancer patients (18 men and 17 women) with ages ranging from 24 to 71 years old were included in this study. Direct interviews with the patients were conducted to obtain clinical data, along with investigations into their hospital records and past medical histories. Control Group: 30 tissue biopsies from autopsies at the forensics medicine department and histopathologically proved free of malignancy which were taken as normal tissue (12 males and 18 females with a mean age of 45.11 years and a range between 21 and 70 years) and were used as controls for evaluation in Immunohistochemistry (IHC). Benign tumor: 35 endoscopic biopsies of polyp (22 female and 13 male with a mean age of 47.6 and a range between 29 and 72) were taken from the diagnostic endoscopy department at the Gastroenterology and Liver teaching hospital. All been histopathological proved to be benign polyposis.

Histological screening

Large intestine tissue samples (biopsy and masses) were obtained, fixed in 10% neutral buffered

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formalin, dehydrated in ethanol series of increasing concentrations (70%, 80%, 90%, 95%), and cleared with xylene before being fixed in paraffin (to form tissue embedded block). In the laboratory of the pathology department in the college of medicine Al-Nahrain University, the blocks were divided into 5 µm thick segments using a microtome. Hematoxylin and Eosin-stained tissue sections were examined under a light microscope for histological examination by consulting histopathologists. Histological changes for adenocarcinoma were confirmed based on H&E staining (biopsy and mass). Two or more paraffin-embedded blocks made from various colon sits were created for each patient. Nevertheless, each patient has at least one block with paraffin-embedded. Hematoxylin and Eosin (H & E) staining sections were reviewed by specialized consultant histopathologists to determine the colorectal carcinoma's histological type and stage. 30 normal colorectal tissue samples were collected as a control from healthy, cancer-free individuals, and 35 polyp tissue biopsy samples were also collected from the endoscopy section at gastroenterology and liver diseases hospital.

Histologic staging: as categorized by the WHO

Table 1: TNM staging of colorectal cancer (6).

Stage		Level of involvement
Tumor	T1	Limited to mucosa and submucosa
	T2	Extension into but not through muscularis propria
	T3	Invasion of perirectal fat
	T4	Invasion of adjacent structures
Nodes	N0	No involved lymph nodes
	N1	Fewer than four regional nodes involved
	N2	More than four regional nodes involved
	N3	Distant nodes involved
Metastasis	M0	No metastasis
	M1	Distant metastasis

Immunohistochemically Procedure

A. Slide preparation: Paraffin-embedded sections were cut into 5µm thickness, then the sections were carried by adhesive positively charged slides, and sections were left to dry to facilitate adhesion between the section and the charged glass surface.

B. Deparaffinization and rehydration: this step involves Dewaxing of paraffin-embedded sections was placed inside a hot air oven at 65°C for 30 minutes. Deparaffinization was done by immersing the slides in xylene for 3 minutes and then in fresh xylene for 5 minutes. Rehydration of tissue section accomplished through immersing of slides in sequential dilutions of ethanol

C. Antigen Retrieval: According to your marker (30 minutes 90 ˚C) in the Chamber Boiling method.

D. Peroxidase block: Slide encircled with Pap pen. Hydrogen peroxide was applied to cover the tissue and incubated for 20 minutes. Then the slides were rinsed with distilled water, drained, and blotted gently.

E. Protein blocking of Non-specific binding of primary antibody: Before adding the primary antibodies, slides were ready for the blocking step, to block endogenous Fc receptors and high-affinity proteins, 100 µl of Reagent 1 was added and incubate in sections for 20 min to prevent any unspecific binding of primary antibody (FC region) with tissue section, this with prevent false positive results. Then slides were drained and blotted without washing.

Immunostaining steps: these multi steps specific interaction between primary antibodies reacts with the target antigen and end by colored designation of the get:

1) 100µl of the f primary antibody was placed onto the tissue section and incubated for 1 hour at 37˚C in a humid chamber. After incubation, the slides were drained and blotted gently. Then Slides were placed in a washing buffer bath for 5 minutes twice, drained, and blotted gently.
2) 100µl of the antibody amplifier was applied to the sections then the slides were placed in the humid chamber and incubated at 37°C for 1 hour. The slides were rinsed and placed in a washing buffer bath before the excess buffer was drained and blotted gently.
3) 100µl of HRP polymer conjugate was placed onto the tissue section and incubated for 1 hour at 37˚C in a humid chamber, then slides were placed in a washing buffer bath for 5 minutes, drained, and blotted gently.
4) 50µl of the DAB-substrate chromogen (20µl of DAB mixed with 1ml of substrate diluent) was placed onto the tissue section and incubated for 5 minutes at 37˚C in a humid chamber, and then slides were rinsed in distilled water, drained and blotted gently.
5) Counterstain: the slides are immersed in a bath of Mayer's Hematoxylin for 1 minute. Slides were washed three times in distilled water, 1 minute each; then drained and blotted gently.

Post staining steps: include preparation of stained slides for microscopic examination:

1. Slides were dehydrated by placing them in Ethanol and Xylene-containing jars in the following order:
2. 50% ethanol for 5 minute.
3. 70% ethanol for 5 minute.
4. 90% ethanol for 5 minutes.
5. Absolute ethanol for 5 minutes.
6. Xylene for 5 minutes.

7. A drop of mounting media was placed onto the section and the tissue section was quickly covered with a cover slip and slides were left to dry.
8. Negative control was included for each run of immunohistochemistry. The slide was not allowed to dry in any step of the immunostaining.

Evaluation of the Immunostaining

Evaluation of IHC results for performed by light microscope (Genex 20, America) at 40X objective lens. The typical results of immunochemical staining counted in. All results counted as a relative percentage of positive cells stained with dark brown color out of the total count of positive and negative cells.

Scoring criteria

The intensity score of the IHC images was used to evaluate the expression of IL-17 in the tissue samples: Visually evaluating the intensity of the staining, a score of 0 was designated as zero expression, 1, 2, and 3 as mild expression, moderate expression, and strong expression, respectively. Each sample was divided into five randomly chosen fields, and the intensity scores were collected. Negative expression was defined as total scores between 0 and 2, weak expression as total scores between 3 and 4, and strong expression as total scores more than 5 (7).

3. Statistical Analysis

The statistical analysis of this case-controlled prospective study performed with the statistical package for social sciences (SPSS) 20.0 and Graph-pad prism Version 7. Numerical data were described as mean and standard deviation and analysis of variance test used for comparison among more than two groups. Categorical data were described as count and percentage. Chi-square test used to estimate the association between variables. The lower level of accepted statistically significant difference is bellow or equal to 0.05 (8).

4. Results

Detection of IL-17A protein

Two histopathological investigators evaluated the score for the detection of immune expression of the IL-17A protein using a semi-quantitative visual grading system. IHC with DAB chromagen and Myer's hematoxylin was used to identify the expression of IL-17A.

The immune-reactive score of IL-17A protein expression was determined semi-quantitatively and the highest frequency of positive cases was in resection tissue (score 1), 17 cases (48.6%) and (score 2) 13 cases of malignant tissue (37.1%), followed by 12 cases (34.3%) of benign tumor tissue in (score 1).

The Chi-square test showed highly significant differences between the expression of IL-17A and the type of tissue with a p-value (p=<0.001).

Table (2): Frequency and scores of IL-17A according to the type of tissue.

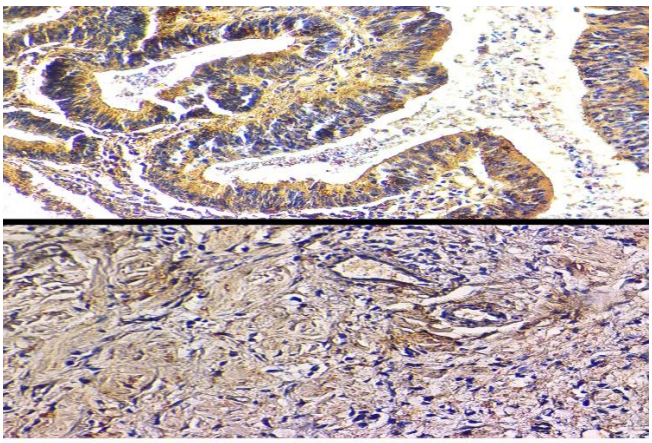
		Group			
		Benign (n=35)	Control (n=30)	Malignant (n=35)	Resection (n=35)
IL-17 A Expression score	0	13	17	0	8
		37.1%	56.7%	0.0%	22.9%
	1	12	12	11	17
		34.3%	40.0%	31.4%	48.6%
	2	9	1	13	10
		25.7%	3.3%	37.1%	28.6%
	3	1	0	11	0
		2.9%	0.0%	31.4%	0.0%
P value		<0.001**			

**: High statistical significance (p≤0.001).

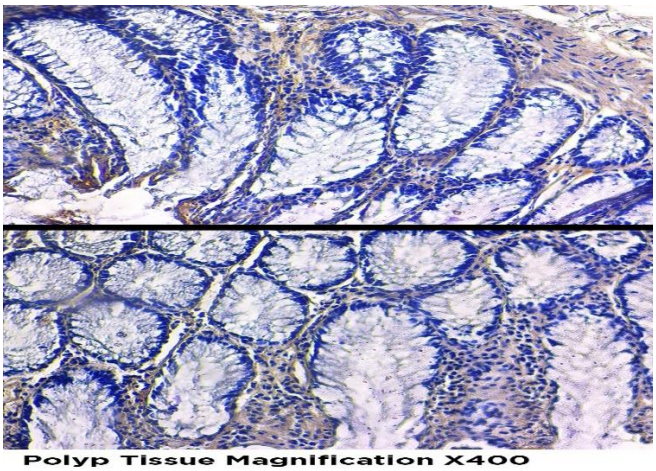
Table (3): Frequency and scores of IL-17A according to the staging of tumor.

		IL-17 A Expression score							
		0		1		2		3	
		Count	%	Count	%	Count	%	Count	%
Tumor	T1	0	0 %	3	50.00%	3	50.00%	0	0.00%
	T2	0	0 %	1	16.70%	3	50.00%	2	33.30%
	T3	0	0 %	3	42.90%	2	28.60%	2	28.60%
	T4	0	0 %	4	25.00%	5	31.20%	7	43.80%
P value		0.533NS							
Nodes	N0	0	0 %	2	28.60%	5	71.40%	0	0.00%
	N1	0	0 %	3	21.40%	6	42.90%	5	35.70%
	N2	0	0 %	6	42.90%	2	14.30%	6	42.90%
P value		0.084NS							
Metastases	M0	0	0 %	5	33.30%	7	46.70%	3	20.00%
	M1	0	0 %	6	30.00%	6	30.00%	8	40.00%
P value		0.414NS							

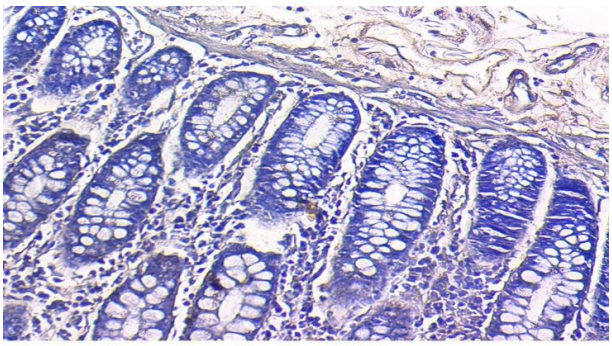
NS: None statistical significance (p>0.05).



Figure(1): Immunohistochemical staining Of IL-17A in CRC section By HRP/DAB (Brown) counter stain with Hematoxyline.



Figure(2): Immunohistochemical staining of IL-17A in polyp section By HRP/DAB (Brown) counter stain with Hematoxyline.



Figure(3): Immunohistochemical staining Of IL-17A in control section By HRP/DAB (Brown) counter stain with Hematoxyline.

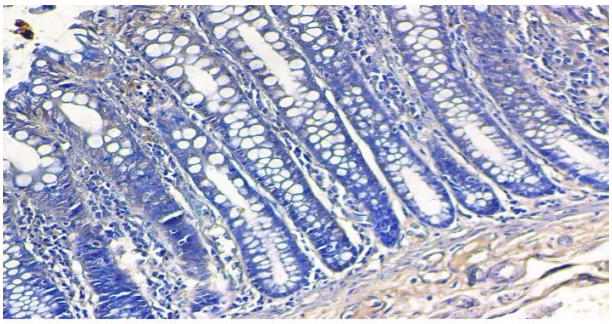


Figure (4): Immunohistochemical staining of IL-17A in adjacent tissue section by HRP/DAB (Brown) counter stain with Hematoxyline.

The IL-17A expression and the cancer stags

According to the staging system it was mostly expressed in patients with extensive tumor invasion (T4) and that appeared in 7 patients (43.80%) compared to patients with T1+T2+T3, with a non-significant P value (P =0.533).
And according to lymph node spreading of cancer, it was mostly expressed in N2 with 6 patients (42.90%) compared to patients with N0+N1, with a non-significant P value (P =0.084).
It was also mostly expressed in the metastases patient at M1 with 8 patients (40.00%), with non-significant P value (P =0.414).

5. Discussion

Th17 cells appear to have two main roles: controlling the intestinal microbiota and getting rid of extracellular bacteria and fungi. It has been demonstrated that IL-17A and IL-17 receptor signaling contribute to the protection of the host against a variety of bacterial and fungal infections (9).
In this study, the expression of IL17A showed significant differences depending on the type of tissue with a p-value (p=<0.001) and with a non-significant P value (P =0.414) according to the different stages of CRC based on the TNM staging system.
These findings agreed with Y Lin et al 2015(10) who found that the Positive IL-17A protein expression was observed more frequently in CRC as compared to colorectal adenoma and non-tumor tissue, respectively (P < 0.01). IL-17A expression correlated to well differentiation and early-stage CRC has also been found the expression of IL17A is highly significant (p<0.01) in CRC patients(104). That might be due to the IL-17A response that acts as a protective mechanism for the body's defense against numerous infections, but it also has a double-edged role as a risk factor for the onset of autoimmune diseases such as Crohn's disease (CD) and ulcerative colitis (UC), the two main forms of inflammatory bowel diseases (IBD) in man and two of the most important risk factors for CRC development (11).

6. Conclusions

1. It has been found that the expression of IL17A is highly significant in CRC patients and it might have a double-edged role as its important in controlling the intestinal microbiota and getting rid of extracellular bacteria, and it pro-inflammatory effect in tumor development.
2. IL17A expression has no significant value according to the different stages of CRC based on the TNM staging system.

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